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Characterizing and Targeting Bone Marrow-Derived Inflammatory Cells in Driving the Malignancy and Progression of Childhood Astrocytic Brain Tumors

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14. ABSTRACT

In this study, we have utilized glioma patients along with two unique murine glioma models: RCAS glioma model and Gl261 model to study various lineages of BMDCs during different stages of glial tumors. Importantly, we identified the unique the population VEGFR2+MDSCs in both patients and mice, which might be used as a surrogate marker for glioma diagnosis and prognosis in future. We have validated the changes of myeloid lineage and endothelial lineages during the progression of gliomas, and We observed bone marrow derived mesenchymal stem cells have only minimal effort on tumor progression. We have created inducible VEGFR2 knockout system in RCAS-tva model. We demonstrated that bone marrow derived VEGFR2 signaling plays an important role in myeloid differentiation, and infiltration into tumor tissues. Deficiency of VEGFR2 in BMDCs led to impairment of tumor associated myeloid cells and delayed progression of low-grade glioma. Primary tumor up-regulates VEGFR2 in myeoloid cells through ID2/E2A pathway. This work has shown the importance of myeloid derived ID2/VEGFR2 signaling in low-grade to high-grade glioma transformation.

15. SUBJECT TERMS

Glioma, Pediatric, bone-marrow-derived-cells, endothelial, mesenchymal, myeloid, hematopoietic, differentiation, malignant, transformation, VEGFR2, ID2.

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1. Introduction

Brain tumors are most frequent solid cancer among all kinds of childhood cancer. Heterogeneity, invasiveness, and complex microenvironment are making therapies as well as research on asrtocytic brain tumor particularly challenging [1,2]. Low-grade gliomas are curable and most patients could live without further progression and severe condition for many years. However, once the glioma progress to high grade, the quality of life and survival of patients are very poor [1]. Based on previous work, we hypothesized the bone marraw derived cells (BMDCs) could serve as a mediator of transition of low grade to high-grade tumor [3-5]. The proposed project aims to characterize the various lineages of glioma associated BMDCs including hematopoietic, endothelial, and mesenchymal lineages in both low grade and high grade stages of glioma. Studying the effect on glioma transition from low grade to high grade by depleting distinct populations of bone marrow derived inflammatory cells including monocyte, granulocytes, endothelial progenitors, and mesenchymal progenitors. Dissecting molecular mechanism/signaling of differentiation of glioma associated BMDCs, and screening the key factors or targets through the entire regulatory pathway. It would contribute to develop therapeutic strategies to target a specific population of BMDCs and their subsequent recruitment, in order to suppress the malignant transformation of gliomas. In this project, we have initiated the study of BMDCs with RCAS and Gl261 murine glioma models as well as glioma patients. We also used transgenetic tools to deplete certain populations of BMDCs to study functional contribution of BMDCs for glioma progression.

2. Keywords

Glioma, Pediatric, bone-marrow-derived-cells, endothelial, mesenchymal, myeloid, hematopoietic, differentiation, malignant, transformation, Inhibitor of DNA binding protein.

3. Overall Project Summary

The project focuses on studying the microenvironment and functions of bone marrow derived cells within pediatric astrocytic tumor. In this study, two major glioma models will be used to investigate the role of BMDCs primarily. One is a transgenic mouse using the RCAS/Tv-a system created by Holland and Varmus that develops low-grade gliomas which progress to high-grade tumors over the course of twelve weeks [5,6]. The other model is syngeneic orthtopic glioma model. Gl261 [7,8], a C57/Bl6 derived glioma cell line with different markers such as GFP or luciferase, was intracranial injected to C57/Bl6 mice to create allograft glioma. In addition, xenograft models with human glioma cell lines are also utilized. Furthermore, we also have used glioma patients' blood samples to analyze various lineages of BMDCs [9-12].

Task 1. Charactering the various lineages of glioma associated BMDCs including hematopoietic, endothelial, and mesenchymal lineages in both low grade and high gradestages of glioma. (100% complete)

We have analyzed the myeloid lineage and endothelial lineage of BMDCs in both patients' blood samples and murine glioma models at low-grade or high-grade stage.

1a. Assess the frequency and absolute numbers of circulating BMDCs in glioma patients. Using flow-cytometry of hematopoietic, as well as endothelial and mesenchymal markers, we will investigate whether the frequency of HPCs, VEGFR2+ EPCs and CD105+ MSCs correlates with glioma transformation in patients. Human subjects involve this study will be radiographically-suspected or biopsy prove low-grade astrocytoma (WHO grade I and II) versus histology proven high grade gliomas (WHO grade III and IV). Blood samples will be collected at the time of diagnosis. Subjects selected for this study may be between 2 years and 16 years of age. We plan to enroll 160 human subjects, and quarterly enrollment is 20. The identifications of human subjects will not be accessible for research team, and only information of human subjects that research team is aware of is the patients' diagnosis and diseases' history.

Up to date, we have recruited 107 glioma patients with low-grade or high-grade glioma, plus 26 healthy volunteers as control into this study. Patients or healthy volunteers' peripheral blood has been analyzed with various lineages makers as showed in the figures below:

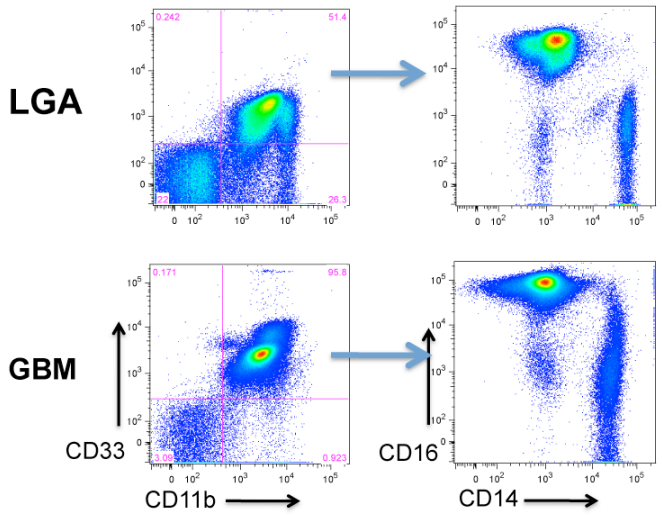


Figure 1-1. Characterizing myeloid lineage of BMDCs in patients (CD11b, CD33, CD14, and CD16) by flow cytometery in peripheral of low-grade astrocytoma patients (LGA) vs glioblastoma patients (GBM).

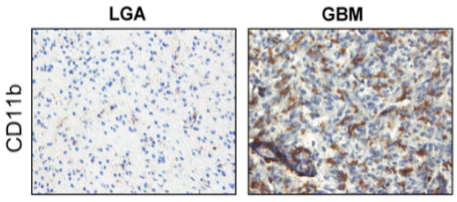


Figure 1-2. IHC of CD11b (infiltrated myeloid cells) on archived paraffin embedded tumor tissue from low-grade astrocytoma patients (grade II) vs glioblastoma patients (grade IV).

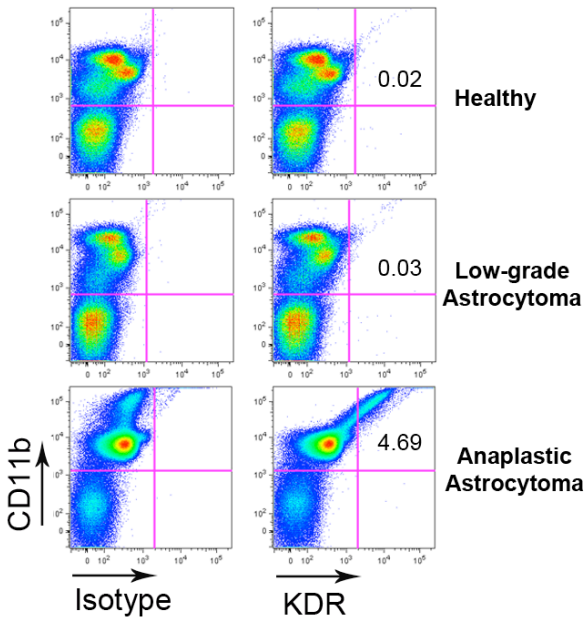


Figure 1-3. Characterizing endothelial/myeloid lineage of BMDCs in patients by CD11b, KDR (VEGFR2) in peripheral of low-grade astrocytoma patients (LGA), glioblastoma patients (GBM), and healthy volunteers.

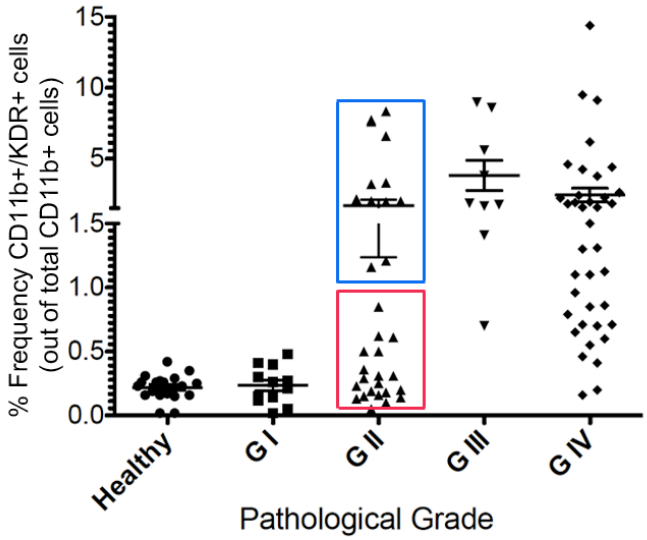


Figure 1-4. Statistic dots-plot on frequency of CD11b+/VEGFR2(KDR)+ cells out of total CD11b+ cells in patients with different stages of disease. Healthy donor served as a control. GIII/GIV vs healthy, One way ANOVA, P <0.001.

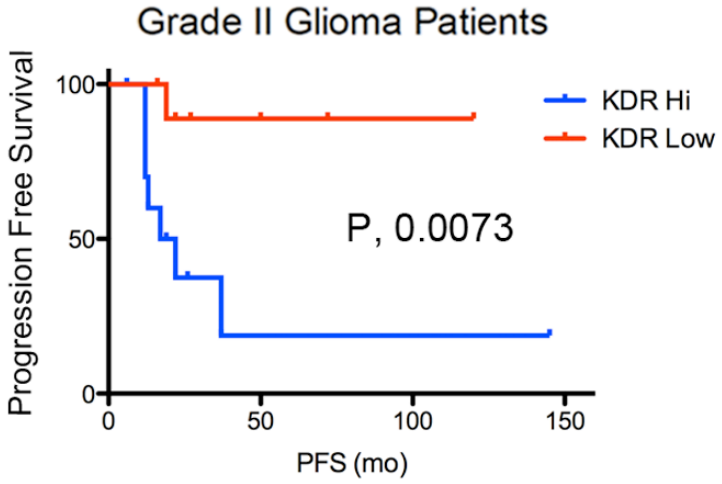


Figure 1-5. Grade II glioma patients were sub-divided into two groups, based on the frequency of CD11b+/KDR (VEGFR2)+ cells: KDR Hi and KDR Lo (cut-off, 1.0%). Kaplan-Meier curves for progression-free survival over 12 months are presented (27 subjects). P <0.01 by log-rank test.

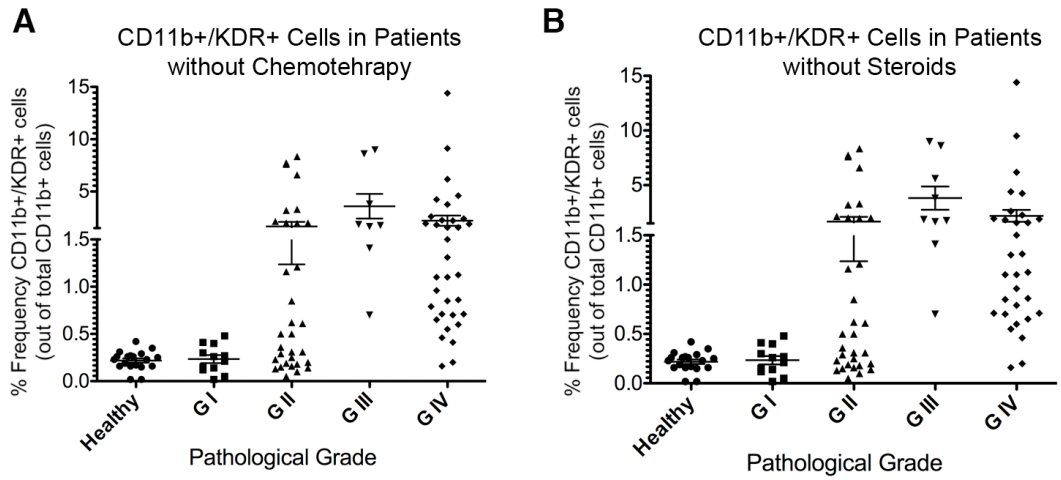


Figure 1-6. Characterize myeloid cells in glioma patients. Dots-plot on frequency of CD11b+/KDR+ cells out of total CD11b+ cells in patients of different stages of glioma without chemotherapy (A) or without Steroid treatment (B).

During the study of BMDCs with glioma patients' blood samples, we have found that the number of myeloid derived suppor cells (MDSCs) within myeloid lineage increased following the progression of diseases. The MDSCs are heterogeneous regarding the expression of CD14/CD16, representing monocytic or granulocytic sub-lineages (Figure 1-1). While the tumor progressed, we also observed more infiltrated myeloid cells within tumor tissues (Figure 1-2). Interestingly, when we examine the expression of VEGFR2 to study endothelial lineage of BMDCs, we found the majority of VEGFR2 expressing cells are CD11b positive as showed in Figure 1-3. It suggested that there is overlap or interaction between myeloid lineage and endothelial lineage of BMDCs. Quantification of CD105+/C-kit+ BMDCs indicates no significant difference in MSCs from patients with in low-grade patients vs high-grade patients. Therefore, we would focus on raveling the role of myeloid/endothelial lineage in glioma progression by following animal study.

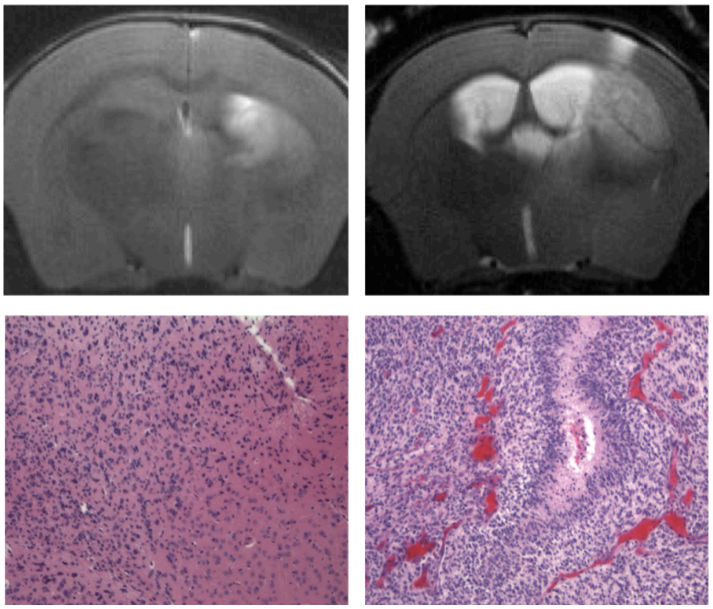
Based on the analysis on large cohort of patients, we found that the number of CD11b+/KDR+ BMDCs have significant correlation with the pathological diagnosis of patients. The ratio of KDR+ myeloid cells was significantly higher in blood samples derived from patients with high-grade gliomas when compared with samples from patients with low-grade gliomas. Specifically, the levels of CD11b+KDR+ cells were higher in patients with WHO grade III or IV tumors than it is in patients with grade I tumors and healthy controls (Figure 1-4). Perhaps most striking is the apparent delineation of two distinct populations of patients, each with pathologically defined grade II astrocytoma. We sub-divided patients with grade II astrocytoma into two subgroups, those with high versus low CD11b+KDR+ values (cut-off of 1.0%). The clinical course and medical histories of the patients were closely followed over 12 months. Disease progression in patients with grade II glioma was assessed by MRI and through subsequent histological diagnoses of grade III or IV glioma. The progression-free survival of our cohort of grade II patients is shown in Fig. 1-2c. Patients with an elevated percentage of CD11b+KDR+ (KDR Hi) myeloid cells presented a significantly higher likelihood of tumor progression from fibrillary to anaplastic variants (Figure 1-5).

To ensure that this finding is not a reflection of differences in the treatment patients received based on the tumor or symptoms. We examined treatments patients received in

each group. Within the patient populations we analyzed, two grade III patients and four grade IV patients had received chemotherapy; seven grade IV patients had received steroids; none had received radiotherapy and targeted therapy before or at the time the samples were collected. Figure 1-6 showed that the statistic significances between each group still remains by excluding patients receiving chemotherapy or steroids.

1b. Evaluate the frequency and number of the same BM-derived populations in blood, bone marrow and metastatic organs of murine models of glioma during low grade, transformation grade and high grade phases. Specifically, we will investigate the mobilization of HPCs, EPCs, and MSCs by flow cytometry.

We have analyzed the HPCs, EPCs, and MSCs in both RCAS and Gl261 murine glioma models. The 4~6 weeks post-injection of RCAS model were consider as the low-grade stage, and 6~9 weeks were considered high-grade stage. The Gl261 model was considered as high-grade glioma model.



Week 5 Week 8

Figure 1-7. RCAS-tva murine glioma model on low-grade stage (week 5) and high-grade stage (week 8). The MRI or H&E staining from representative mice were showed.

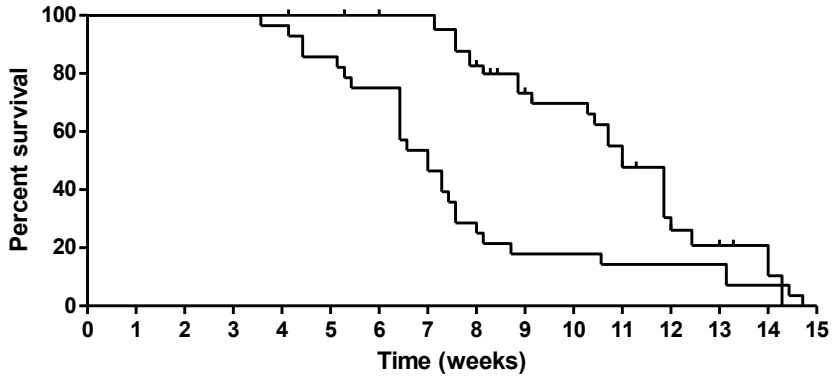


Figure 1-8. The Kaplan-Miere Survival curves of RCAS-tva tumor bearing mice with or without bone marrow transplantation (BMT). The median survival time for RCAS mice without BMT is 7 weeks, and with BMT is 11 weeks.

We characterized the RCA-tva murine glioma model in our experimental setting, and figured out their low-grade stage and high-grade stage evidenced by MRI and histology. Additionally, we showed the RCAS mice with bone marrow transplantation have delayed progression of tumor, which is important for our next step of study.

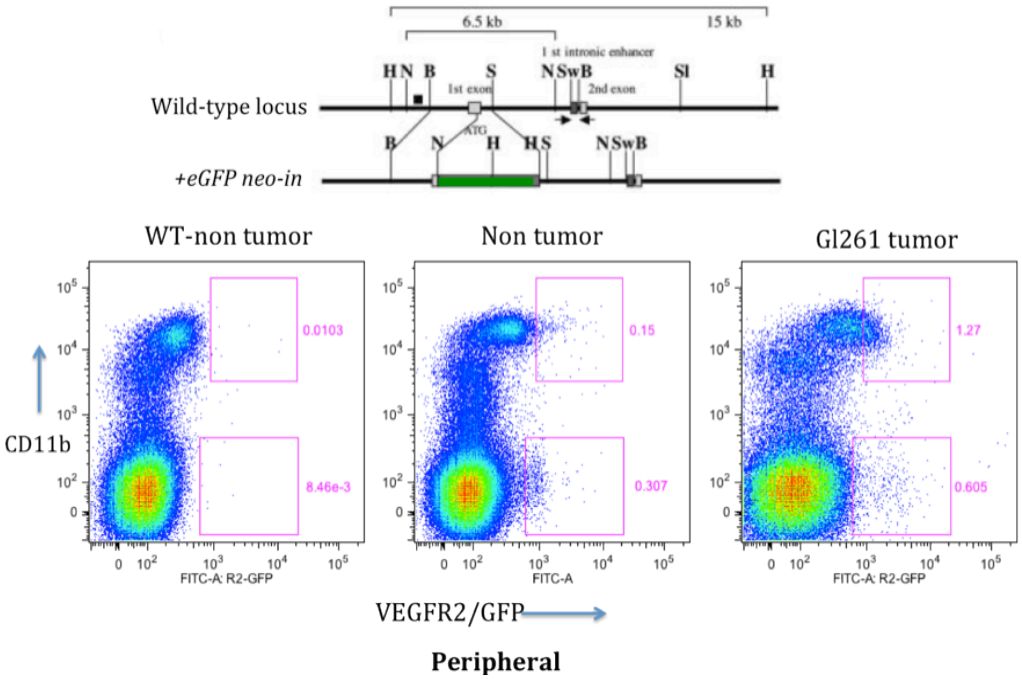


Figure 1-9. The expression of VEGFR2 on BMDCs in Gl261 model. Upper panel of schematic model showed the VEGFR2-GFP knock in mice for studying expression pattern of VEGFR2. Lower panel of flowcytometry graphs indicate the expression VEGFR2 on CD11b+ or CD11b- population.

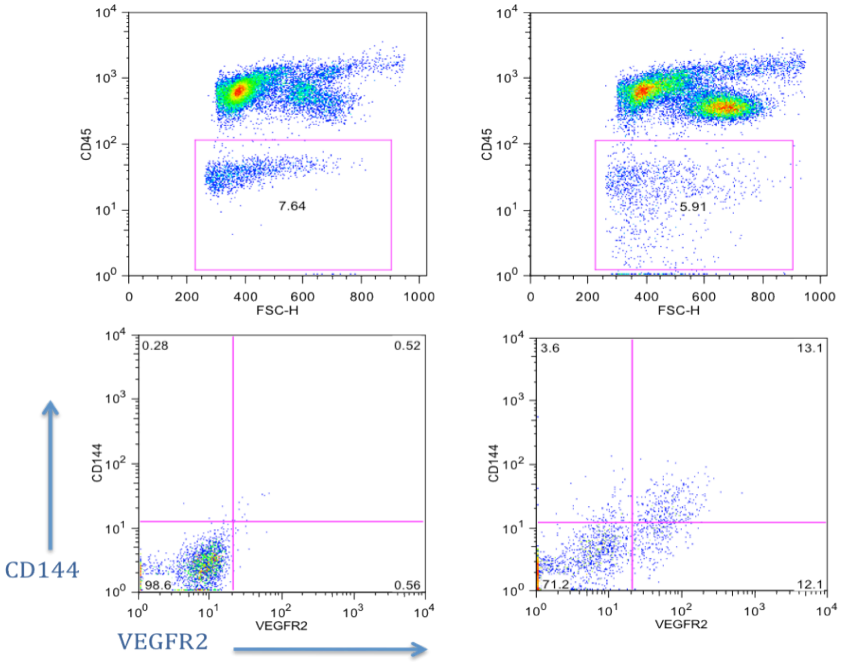


Figure 1-10. EPC in Gl261 glioma model. Flowcytometry graph of lineage negative CD144+ VEGFR2+ EPC in Gl261 tumor bearing mice.

We have studied the myeloid lineage and EPC with Gl261 glioma model. We observed that both VEGFR2+ myeloid cells and EPCs were elevated by Gl261 tumor (Figure 1-9 and Figure 1-10), indicating potential interaction of BMDCs differentiation with primary glioma.

Task 2. Studying the effect on glioma transition from low grade to high grade by depleting distinct populations of bone marrow derived inflammatory cells including monocyte, granulocytes, endothelial progenitors, and mesenchymal progenitors. (100% complete)

We have set up all trans-genetic mice lines including ITGAM(CD11b)-DTR/EGFP mice, RosaCreERT2/PDGFRαloxP/loxP mice, and RosaCreERT2/VEGFR2loxP/loxP mice in suitable genetic background for bone marrow transplantation experiments. We performed the lineage depletion experiments with ITGAM(CD11b)-DTR/EGFP and RosaCreERT2/VEGFR2loxP/loxP mice for myeloid or endothelial lineages in both RCAS and Gl261 tumor models.

2a. We plan to transplant the bone marrow from ITGAM(CD11b)-DTR/EGFP mice into the RCAS and Gl261 glioma models, and use diphtheria toxin to induce depletion of myeloid cells in this RCAS glioma model.

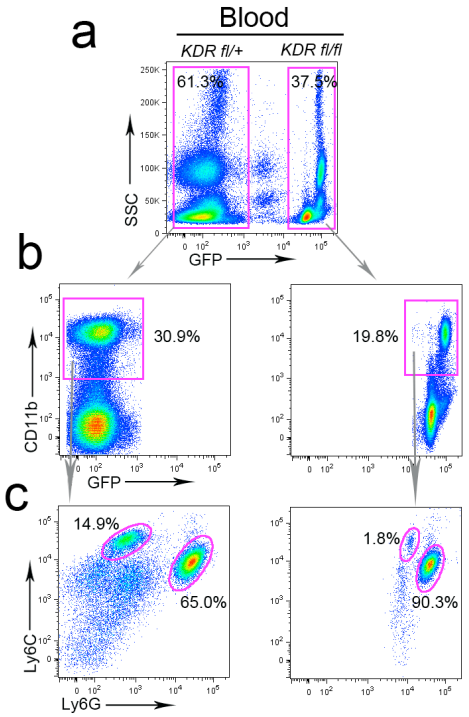


Figure 2-1. Competitive BMT showed that KDR knockout in bone marrow cells lead to deficiency of the differentiation of monocytic cells. Lethal dose irradiated C57/bl6 mice were transplanted with UBC-GFP/rosa26ERT2-cre/KDRfl/fl and rosa26ERT2-cre/KDRfl/+ bone marrow cells, and Gl261 tumors were implanted after bone marrow engraftment. (a) Peripheral white blood cells were analyzed on SSC and GFP by flow cytometry. The GFP+ and GFP- populations were gated for further analysis. CD11b vs GFP (b), Ly6C vs Ly6G (c).

We have developed the ITGAM(CD11b)-DTR/EGFP bone marrow transplanted mice and implanted mice with Gl261 tumor after bone marrow was engrafted. We have tried to deplete the CD11b positive cells in tumor bearing mice once with 5 mice in each group. However, we only obtained approximately 10% deduction of CD11b cells compared with control group. In this case, we didn't see effect on tumor growth. However, as Figure 2-1 showed, knockout KDR (VEGFR2) led to significant reduction of CD11b+ cells, among of which Ly6 C+ cells were suppressed dramatically. In conclusion, knockout VEGFR2 has considerably effect on myeloid lineage, which is inconsistent co-expression VEGFR2 and CD11b in high-grade patients' BMDCs samples. Accordingly, we have used VEGFR2 deficient model in the following study.

2b. Bone marrow from RosaCreERT2/VEGFR2loxP/loxP mice will be transplanted to both RCAS glioma and Gl261 bearing mice to deplete endothelial lineage of bone marrow derived cells by knocking out the VEGFR2 gene. The total number of mice will be used is 30.

We have successfully knockout VEGFR2 in both RCAS model and Gl261 murine model. We have studied the effect of VEGFR2 deficiency on glioma progression.

As Figure 2-2 showed, we could obtain around 90% knocking out efficiency with our RosaCreERT2/VEGFR2loxP/loxP system, without affecting counts of blood cells (CBC) of mice.

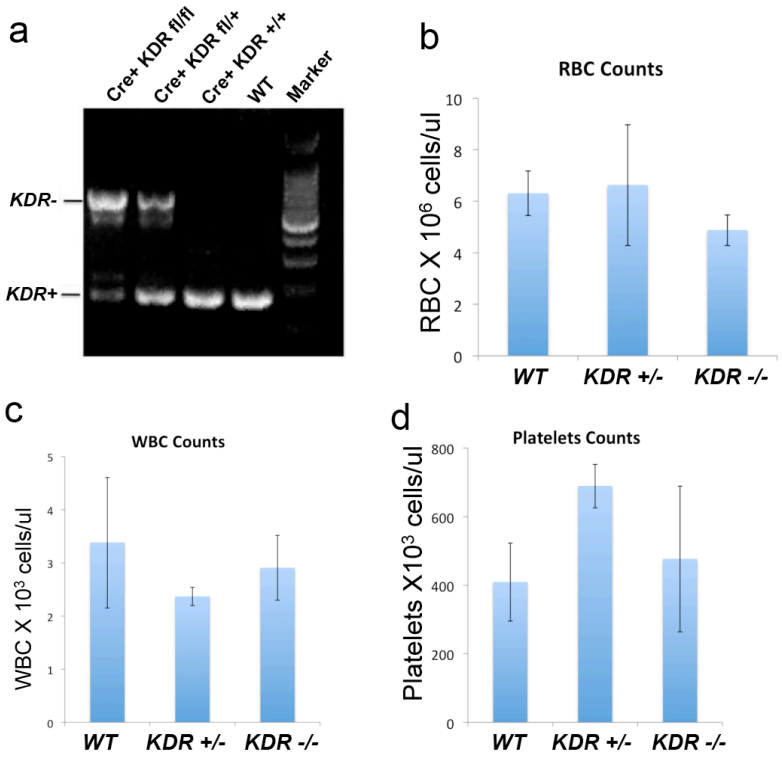


Figure 2-2. Validation of depletion of VEGFR2 (KDR) allele in bon marrow cells. (a) Amplification of KDR+ or KDR- allele on bone marrow cells of mice bearing indicated genetic background. Tamoxifen was applied to mice one week before testing. Complete bloods count on mice with WT, KDR+/-, or KDR-/- bone marrow (b) Red blood cells, (c) white blood cells, and (d) Platelets.

When we knockout VEGFR2 from BMDCs in Gl261 models, we observed that tumor progression were suppressed as showed in figure 12. In the tumor tissue, we found much less tumor associated myeloid cells (CD11b+) in the VEGFR2 KO group compared with control group (Figure 2-4). With RCAS system, we found the similar phenotype, after we performed the bone marrow transplantation and induced VEGFR2 knockout, we observed that tumor progression were delayed and median survival time (MST) was significantly elongated (Figure 2-5).

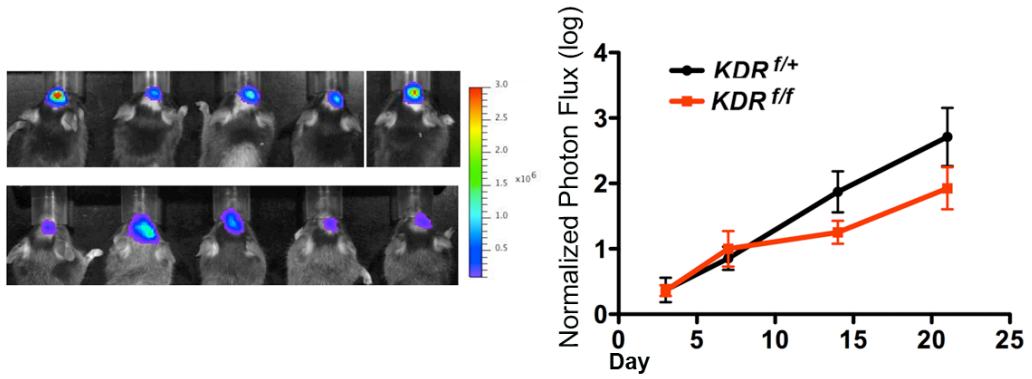


Figure 2-3. Knocking-out VEGFR2 (KDR) in BMDCs suppress Gl261 tumor growth, tumor-associated myeloid cells, and vasculatures. Chimeric C57/bl6 mice transplanted with rosa26ERT2-cre/KDRfl/fl bone marrow cells (labeled as BM-KDR KO, and BM-KDR control is rosa26ERT2-cre/KDRfl/+) were implanted with luciferase labeled-Gl261 tumors intracranially. Tamoxifen were applied at day 3 post- implantation. The tumors were monitored by bioluminescence. The quantification of bioluminescence based tumor growth.

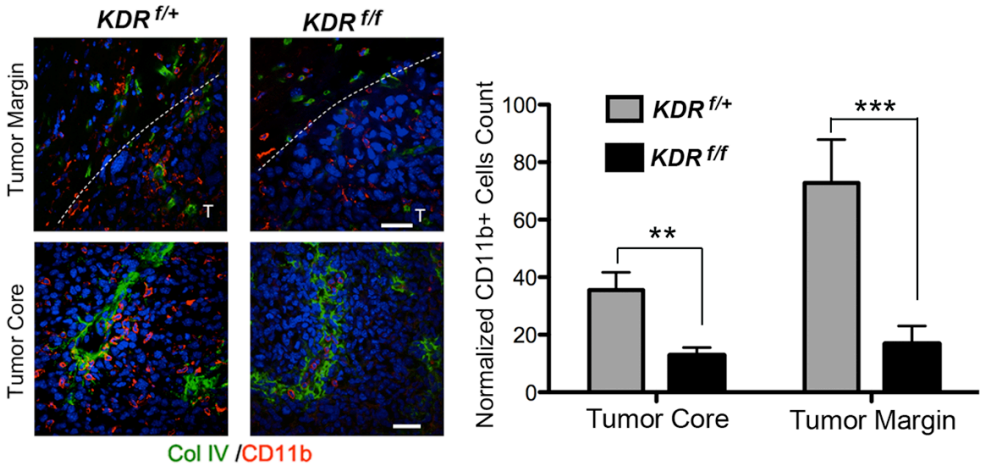
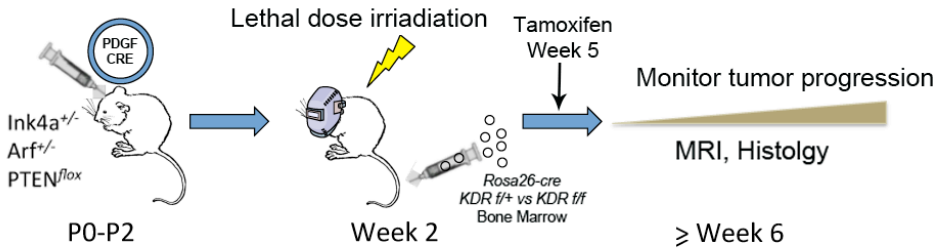


Figure 2-4. Immuno-staining of vascular basement (Collagen IV) and myeloid cells (CD11b) in the Gl261 tumors from each group. Scale bar, 20 μm. The number of CD11b cells in tumor core or tumor margin was quantified.



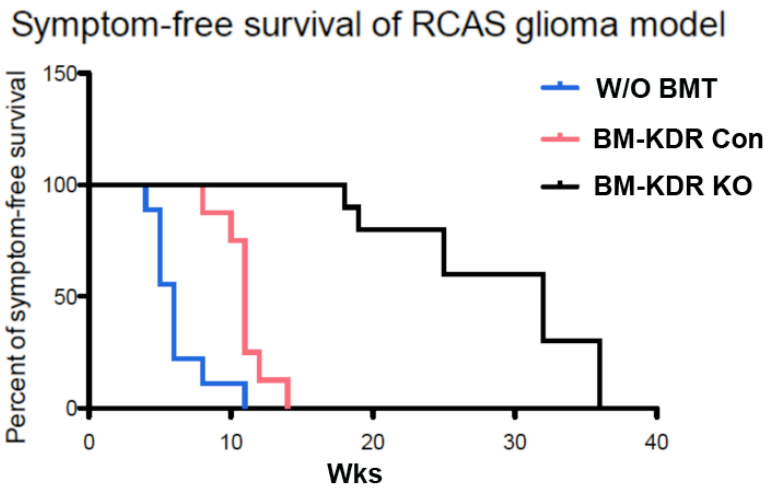


Figure 2-5. Knocking-out VEGFR2 (KDR) in BMDCs suppress spontaneous malignant transformation of RCAS gliomas. (a) Flow chart of experimental design. Oncogenes were transduced to P0-P2 pups in the beginning. Mice received lethal dose irradiation and received bone marrow transplantation at week 2, and then tamoxifen was applied to mice at week 5 to induce the ablation of target gene. Tumors were monitored by MRI over the process. (b) Kaplan-Meier symptom free survial curve for RCAS mice transplanted with rosa26ERT2-cre/KDRfl/fl bone marrow cells (BM-KDR KO), rosa26ERT2-cre/KDRfl/fl bone marrow cells (BM-KDR Con), or without irradiation/transplantation (W/O BMT).

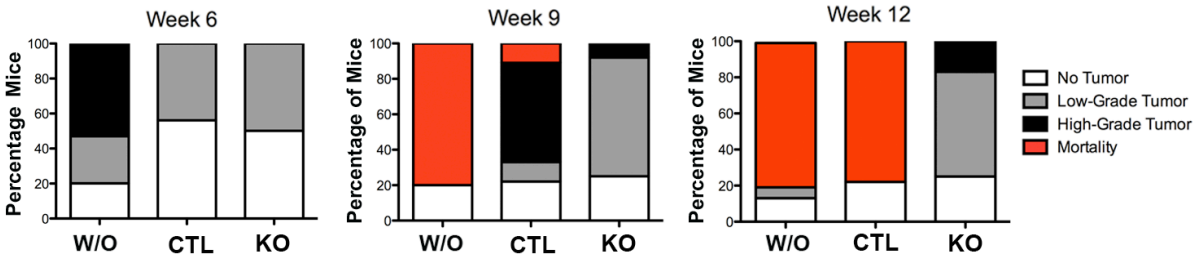


Figure 2-6. At weeks 6, week 9, and week 12, tumors in each group of mice were assessed and graded based on their MRI and further confirmation with histology. N=19~26.

We compared brain tumors in RCAS/tva mice at weeks 6, 9 and 12, utilizing MRI followed by histological examination (Figure 2-6). Knocking out KDR in BMDCs significantly delayed the malignant transformation of low-grade glioma. By week 9, 60% of RCAS/tva mice transplanted with control R26CreERKDRfl/+ bone marrow developed high-grade tumors, which had typical malignant features, including robust gadolinium enhancement on MRI, pseudopalisading necrosis, and microvascular proliferation. In contrast, tumors in 70% of RCAS/tva mice transplanted with R26CreERKDRfl/fl bone marrow remained in the low-grade stage without signs of malignant transformation at week.

2c. Bone marrow cells from RosaCreERT2/PDGFRαloxP/loxP mice will be transplanted to each genetic and orthotopic glioma generating mice. PDGFRα is expressed on bone marrow derived mesenchymal stem cells and PDGF- PDGFRα signal axis is very critical for maintenance of mesenchymal lineage. By knocking out

PDGFRa gene, we will study the influence of defection of mesenchymal differentiation on the progression of low-grade glioma. The total number of mice will be used is 30.

We have completed the crossing donor RosaCreERT2/PDGFRαloxP/loxP mice, and the bone marrow transplantation experiments along with tumor study are ongoing. As study has showed, Deletion of PDGFRa gene in BMDCs have no significant influence on development of Gl261 tumor in mice brain. Interestingly, we observed that tumor did growly slower and in RosaCreERT2/PDGFRαloxP/loxP mice compared with control mice. It suggests that residential PDGFRa+ cells play more important role in tumor progression than bone marrow derived PDGFR+ cells. (Figure 2-7)

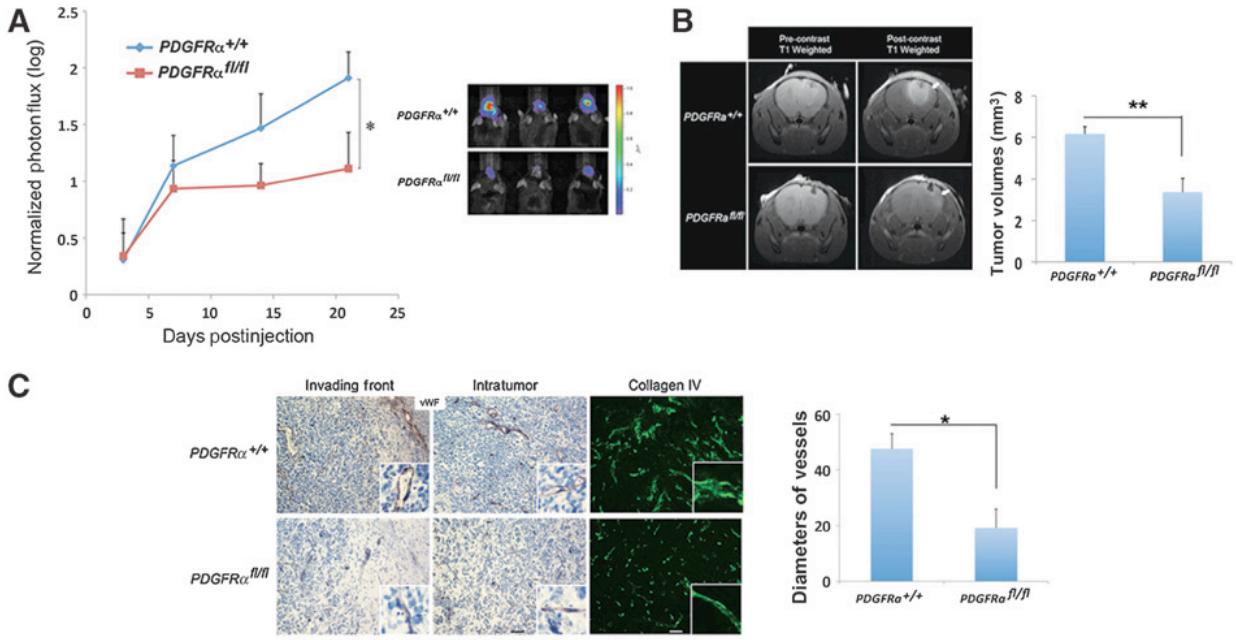


Figure 2-7. Knocking out PDGFRa in stromal cells inhibits glioma progression. Luciferase-Gl261 cells were intracranially injected into RosaCre-ERT2/
PDGFRa fl/fl mice and RosaCre-ERT2/PDGFRa+/+ mice. A, tumor burden is represented by photon flux intensity. $P < 0.05$. B, precontrast and postcontrast T1-weighted MRIs were performed on each group at day 15. Arrows, enhancing tumor. Tumor volume quantification is demonstrated. $P < 0.01$. C, vWF was stained in the tumor periphery and within the tumor of each group (left and middle panels). Collagen IV was stained in each group (right). Scale bar, 50 μm. Vessel diameters were quantified. $P < 0.05$.

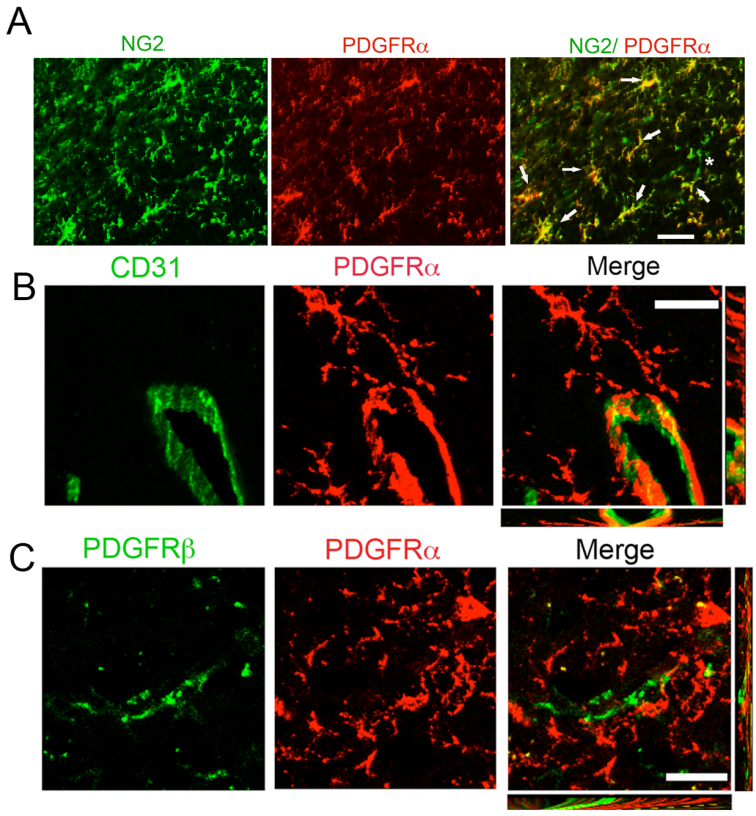


Figure 2-8. PDGFRα stromal cells derived from OPCs. (A) PDGFRα+ cells are OPCs. PDGFRα was co-localized with NG2 in the tumor periphery. Scale bar, 50 μm. (B) PDGFRα+ cells are adjacent to endothelium. PDGFRα was adjacent to blood vessel marker CD31 and is not co-localized with pericyte marker PDGFRβ (C). Z-stack imaging was utilized with confocal microscopy. Scale bar, 20 μm.

Further study has shown depletion of PDGFRα affects oligodendritic progenitor cells (OPCs) in glioma stroma (Figure 2-8). OPCs appears to distribute in perivascular niche and support tumor associate endothelium as pericytes. Deficiency in PDGFc/PDGFα signaling would result in suppression of tumor angiogenesis.

Task 3. Dissecting molecular mechanism/signaling of differentiation of glioma associated BMDCs, and screening the key factors or targets through the entire regulatory pathway. (100% complete)

We will proceed to study which genes in certain lineages of BMDCs could play a critical role in promoting the invasion of glioma. We will isolate specific population from BMDCs from the tumor tissue, blood, and bone marrow. Then we utilize microarray/next generation seuqencing, antibody array, LC-TOF-TOF to detect gene and protein expression. To determine the functional contribution of certain genes of interest, or certain subpopulation of BMDCs, we plan to utilize a series of in vitro/in vivo experiments, including basement invasion assay and knock specific genes bone marrow cells and then transplant them into glioma producing mice, to elucidate their specific roles in promoting invasion of glioma cells and infiltration of glioma. Total number of mice will be used for this study is 30.

We have been working on optimizing sorting different lineages of BMDCs by FACS or MACS and tested a few samples by RNA sequencing. The data suggested that ID2/VEGF2 signaling was playing important role in myeloid differentiation.

In order to further delineate the signaling network driving myeloid/endothelial lineage differentiation, we performed gene expression profiling of VEGFR2-expressing hematopoietic progenitor cells (Lin-C-kit+) by mRNA sequencing. Differentially expressed genes from VEGFR2+ versus VEGFR2- HPCs were clustered and arranged in a heatmap. Differentially expressed genes were also clustered and displayed (Figure 3-1). Candidate genes (P <0.05, >1.5-fold change) were divided according to subsets with the highest expression and analyzed for categories with significant enrichment (P< 0.05) of categories in Gene Ontology (GO) biologic processes using DAVID tools. Similar categories were grouped accordingly (Figure 3-2). Inhibitor of DNA binding proteins 2 (ID2) was identified as a significantly up-regulated gene in VEGFR2+ HPCs a strong candidate to be an upstream molecule mediating myeloid endothelial differentiation.

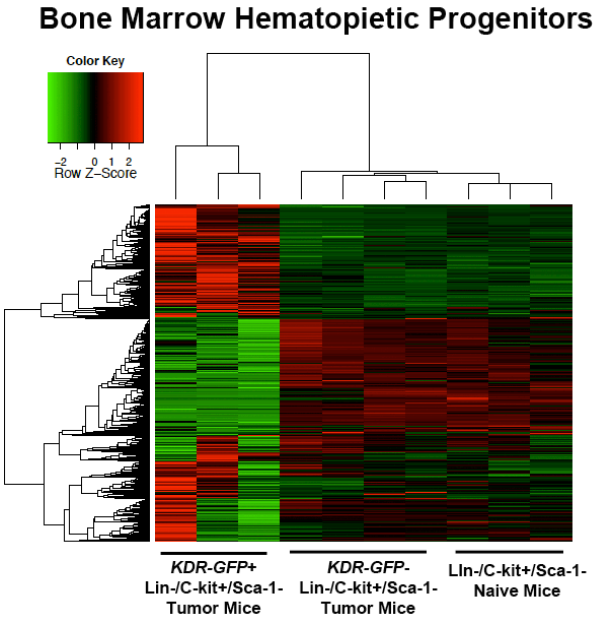


Figure 3-1. RNA-sequencing based gene profiles on KDR+ (VEGFR2) peripheral and bone marrow cells. The heatmap of significantly altered genes in Lin-c-kit+Sca-1- from naïve mice, Lin-c-kit+Sca-1- KDR-GFP+ cells and Lin-c-kit+Sca-1-KDR-GFP- cells from bone marrow of tumor bearing mice.

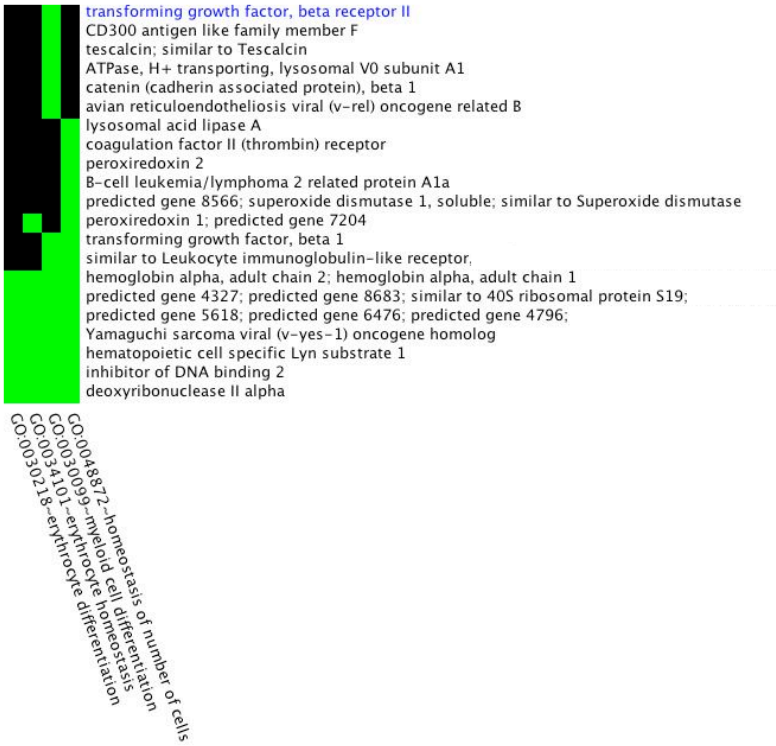


Figure 3-2. The candidate genes (P <0.05, >1.5-fold change; Lin-c-kit+Sca-1-KDR(VEGFR2)-GFP+ vs Lin-c-kit+Sca-1-KDR-GFP-) were divided according to the subset with the highest expression and analyzed for categories with significant enrichment (P< 0.05) of categories in Gene Ontology (GO) biologic processes using DAVID tools. Similar categories were grouped accordingly.

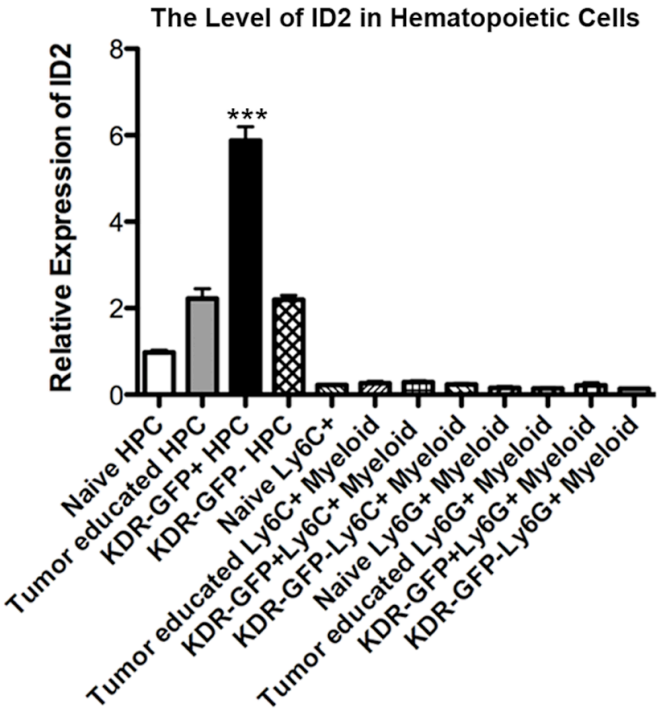


Figure 3-3. The expression of ID2 in various lineages of hematopoietic cells. Means ± SEM, ***P< 0.0001 by one way ANOVA.

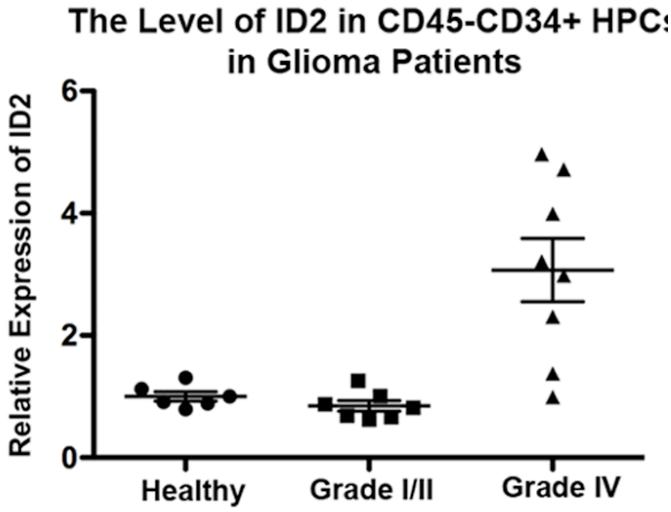


Figure 3-4. The expression of ID2 in hematopoietic progenitor cells (CD45-CD34+) from patients with low-grade or high-grade gliomas. Means ± SEM, P< 0.0001 by one way ANOVA.

To validate the up-regulation of ID2 in HPCs, we performed real-time PCR on KDR^{GFP+} HPCs and $Ly6C^{+}/Ly6G^{+}$ myeloid cells from tumor-bearing or naïve mice. KDR^{GFP+} HPCs from tumor-bearing mice had higher ID2 levels than KDR^{GFP-} HPCs and HPCs from naïve mice (Figure 3-3). The expression of ID2 was generally lower in myeloid cells than in HPCs. We also examined the expression of ID2 in glioma patients. HPCs were isolated from healthy controls and patients diagnosed with low-grade and high-grade gliomas. HPCs from high-grade glioma patients had significantly higher levels of ID2 than did HPCs from healthy controls and low-grade glioma patients (P<0.0001, Figure 3-4).

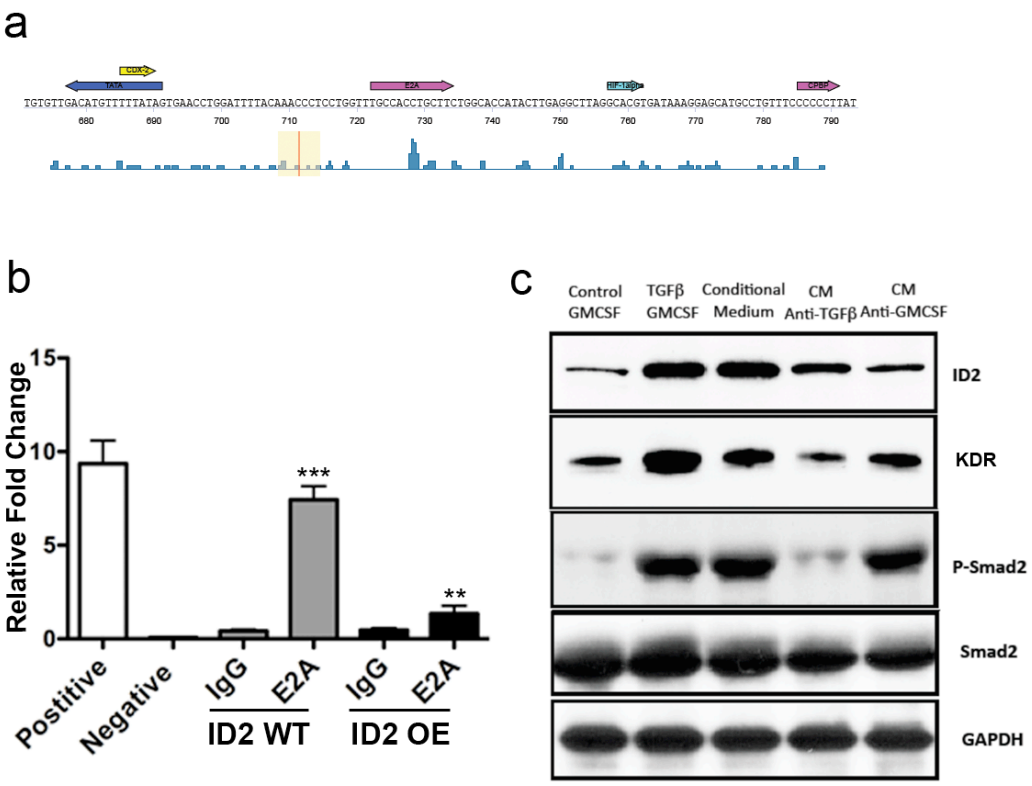


Figure 3-5. Upstream of KDR(VEGFR2) (A) the bioinformatic analysis of promoter and 5'-UTR region of KDR by biobase. Highlighted fragment of KDR promoter showed potential binding site for E2A. (B) Chromatin immunoprecipitation of E2A with or w/o over-expression of ID2. Probe on KDR promoter fragments were used. Means ± SEM, **P<0.01, ***P<0.0001 by one way ANOVA. (C) Immunobloting of KDR and ID2 on in vitro cultured Lineage negative bone marrow cells treated with or w/o TGF-β1 and GMCSF. Phospho-smad2/smad2 indicated activation of related signaling. The immunobloting has three replicates.

ID2 functions by inhibiting the binding to DNA of E proteins. To investigate whether ID2 interacted with E proteins to regulate cell fate within the myeloid lineage, we analyzed the promoter region plus the 5' untranslated region of KDR for putative E protein binding sites known as E boxes. We identified a putative binding site for E2A (Figure 3-5a) within the upstream promoter region of the KDR gene. To conclusively establish the ID2/E-protein/KDR interaction, we performed a chromatin immunoprecipitation assay using E2A antibody in ID2+/+, ID2 over-expressing, and ID2-/- bone marrow cells. As expected, antibody to E2A was able to pull down the upstream fragment of KDR. Over-expression of ID2 abolished the binding of E2A to the promoter of the KDR gene (Figure 3-5b). Although it was beyond the scope and focus of our analysis, our data suggested that soluble GM-CSF and TGF-β may influence the expression of ID2 within pluri-potential bone marrow populations, regulating KDR expression thereby directing cell fate. GM-CSF and TGF-β induced expression of ID2 and KDR in lineage negative bone marrow cells (Figure 3-5c) similar to conditioned medium derived from Gl261 cells; blocking either one reversed ID2 and KDR expression. These results support the hypothesis that glioma-secreted GM-CSF and TGF-β initiate pro-tumoral myeloid cell differentiation, directing the formation of the KDR+-

pro-angiogenic immune cell population, which plays a crucial role in the tumor microenvironment and malignant glioma transformation.

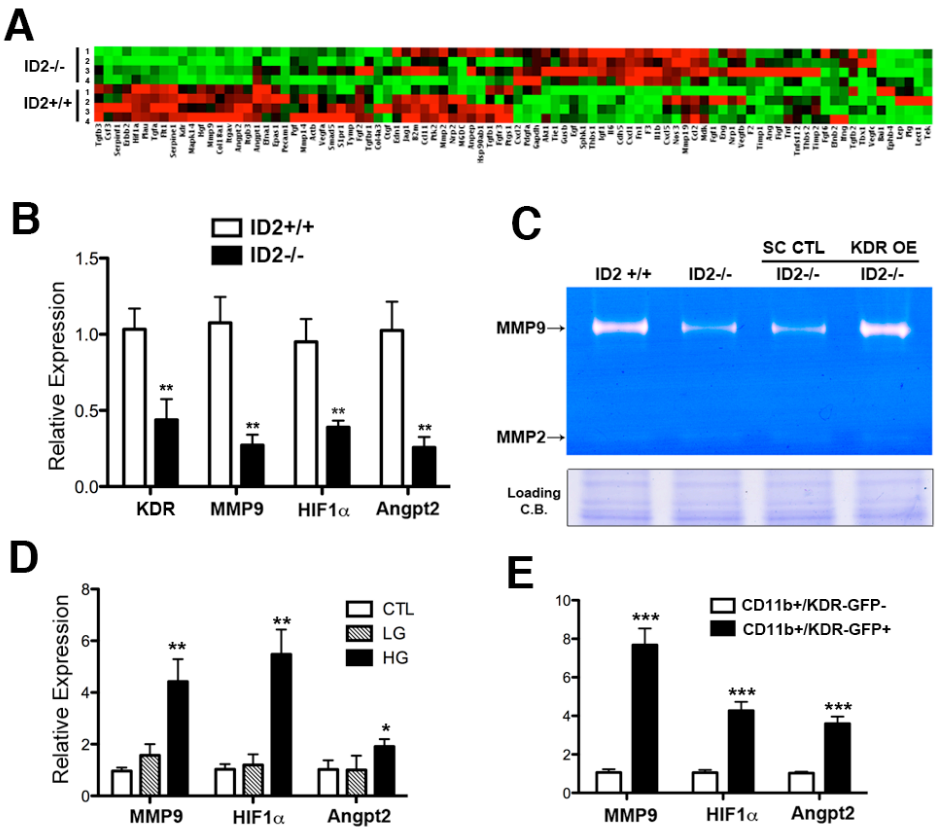


Figure 3-6. ID2/KDR (VEGFR2) affects angiogenic pathway. (A) Quantitative RT-PCR array of multiple pro/anti-angiogenic factors were performed on lineage negative ID2+/+ or ID2-/- hematopoietic Lin- cells treated with TGFβ/GMCSF. Deficient ID2 down-regulates the expression level of KDR. (B) Validation on expressions of KDR (VEGFR2), MMP9, Hif1α, and Angiopoietin-2 by quantitive RT-PCR. (C) Zymography of gelatin gels represented the enzymatic activities of MMP9/MMP2 from condition medium of lineage negative ID2+/+, ID2-/-, ID2-/- SC CTL (transduced with scramble lentivirus), or ID2-/- KDR OE (transduced with KDR overexpression lentivirus) HPCs pre-treated with TGFβ/GMCSF. n=3. (D) The expression of MMP9, Hif1α, and Angiopoietin-2 in CD11b+ blood cells from control (CTL), low-grade glioma (LG) and high-grade glioma (HG) PDGF-RCAs mice were examined by quantitive RT-PCR. (E) The expression of MMP9, Hif1α, and Angiopoietin-2 in CD11b+/KDR-GFP- or CD11b+/KDR-GFP+ blood cells from high-grade glioma (HG) PDGF-RCAs mice. Means ± SD, *P<0.05, **P< 0.01, ***P<0.001 by student's t-test.

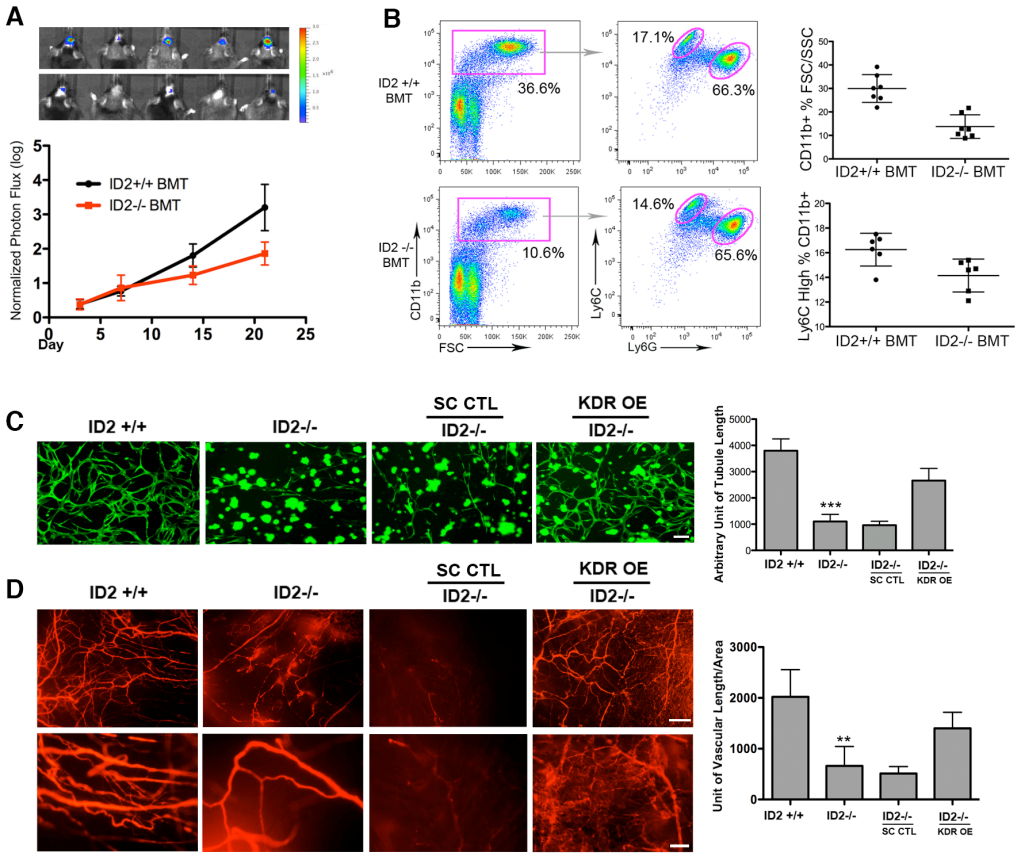


Figure 3-7. ID2 promotes pro-angiogenic phenotypes of tumor-associated myeloid cells. (A) Chimeric C57/BL6 mice transplanted with ID2-/- bone marrow cells (ID2+/+ bone marrow cells as control) were implanted with luciferase labeled-GL261 tumors intracranially. Tamoxifen were applied at day 3 post-implantation. The tumors were monitored by bioluminescence. Representative image was taken at day 14 and color bar on the right represents photon intensity. The experiments have two replicates. The tumor growth curve based on bioluminescence. Means ± SD, n=10, P<0.01 by one way ANOVA (B) Flow cytometry analysis of peripheral blood cells on CD11b, Ly6C, and Ly6G in ID2+/+ BMT and ID2-/- BMT group. Quantification of frequency of CD11b+ cells out total white blood cells and frequency of Ly6Chigh cells out of CD11b+ cells. P in each group < 0.01 by one way ANOVA. (C) Tubule formations of HCMEC/D3 (GFP), co-cultured with lineage negative ID2+/+, ID2-/-, ID2-/- SC CTL, or ID2-/- KDR(VEGFR2) OE HPCs pre-treated with TGFβ/GMCSF. Scale bar, 20 μm. Quantifications of tubule lengths in the indicated group. Means ± SD, ***P< 0.0001 by one way ANOVA. (D) Growth-factors-reduced Matrigel plugs of lineage negative ID2+/+, ID2-/-, ID2-/- SC CTL, or ID2-/- KDR(VEGFR2) OE HPCs pre-treated with TGFβ/GMCSF. Blood vessels (red) were perfused with Rhodamine-Dextran. Upper scale bar, 50 μm. Lower panels are magnified views to highlight vascular permeability, scale bar, 20 μm. Quantifications of based blood vessels density (BVD) in each group. Means ± SD, **P<0.01 by one way ANOVA.

In order to further understand the contribution of the ID2 signaling axis in regulating the differentiation of pro-tumoral and angiogenic myeloid cells within the context of glioma immunomodulation. To begin this analysis, C57/BL6 mice, which received ID2+/+ or ID2-/- bone marrow transplantation, were orthotopically implanted with GL261 tumors. GL261 astrocytic tumors grew more slowly in mice with ID2-/- bone marrow compared

to those with ID2+/+ bone marrow ($P<0.01$, Figure 3-7A). Fewer CD11b+ myeloid cells and, in particular, fewer CD11b+Ly6C+ cells were recruited to tumors in mice with ID2-/- bone marrow compared to those with ID2+/+ bone marrow ($P<0.01$, Figure 3-7B).

To detect candidate downstream targets of ID2 pathway signaling, we employed RT-PCR, utilizing in vitro differentiated BMDCs from ID2-competent or ID2-incompetent tumor-bearing animals. Among target genes identified in this assay (Figure 3-6A), we observed down-regulation of MMP9, HIF1α, and Angiopoietin-2 along with VEGFR2 in ID2-/- BMDCs compared with ID2+/+ BMDCs ($P<0.01$, Figure 3-6 B, C). As Figure 3-6 D showed, MMP9, HIF1α, and Angiopoietin-2 were up-regulated in CD11b+ blood cells of mice with high grade tumor compared with those with low-grade tumor. These pro-angiogenic factors are also significantly higher in KDR positive myeloid cells than those in VEGFR2 negative myeloid cells (Figure 3-6E). To further define and demonstrate the pro-angiogenic functions of the ID2/VEGFR2 pathway within our identified myeloid cell population, we performed in vitro tubule assays under different conditions. We co-incubated eGFP-brain endothelial cells (HCMEC) with previously in vitro differentiated ID2+/+ and ID2-/- BMDCs. In addition, to examine whether VEGFR2 overexpression would have a discernable effect on myeloid cells in the absence of ID2, ID2-/-, BMDCs were also transduced by lentivirus over-expressing either a scrambled control or the wildtype VEGFR2 gene. ID2-expressing BMDCs strikingly promoted tubule formation; however, ID2-deficient BMDCs were unable to support the formation of endothelial tubules. KDR over-expression allowed BMDCs to regain their pro-tubule promoting capability ($P<0.0001$, Figure 3-7C). In parallel, we performed an in vivo matrigel plug assay utilizing the same BMDC conditions. ID2+/+ BMDCs demonstrated potent induction of neo-vascularization in matrigel plugs ($P<0.01$, Figure 3-7D), whereas loss of ID2 compromised the pro-angiogenic functions of BMDCs. Once again, over-expression of VEGFR2 rescued the pro-angiogenic phenotype of BMDCs. Interestingly, while the over-expression of VEGFR2 in ID2-/- BMDCs showed enhanced pro-angiogenic capability, the blood vessels induced by ID2-/- BMDCs with over-expression of VEGFR2 were noticeably leakier when compared with those induced by wildtype BMDCs, as was evidenced by permeability of dextran. While we demonstrated that over-expression of VEGFR2 in BMDCs convincingly rescues the angiogenic deficiency induced by loss of the ID2 gene, the architecture remains imperfect.

4. Key Research Accomplishments

I. We have demonstrated that myeloid derived suppressor cells increased following the progression of astrocytic tumor in both patients and murine models.

II. We identified a specific population across endothelial and myeloid lineages, which is VEGFR2+CD11b+ population in patients and tumor bearing mice.

III. We successfully performed knock out VEGFR2 wihin BMDCs in murine glioma models, and observed that bone marrow derived VEGFR2 contribute to tumor progression and animal survival.

IV. We have performed RNA-sequencing on tumor associated myeloid progenitors, and identified inhibitor of DNA binding proteins 2 was related with pro-tumoral myeloid differentiation.

V. We have demonstrated that ID2 was up-regulated in tumor primed hematopoietic progenitor cells in both patients' samples and murine models.

VI. We identified the binding region of E2A in VEGFR2 promoter, which indicates role of complex E2A/ID2 in regulation of VEGFR2 expression.

VII. Targeting ID2/VEGFR2 axis could restrict the generation of tumor-associated myeloid cells and inhibit the tumor angiogenesis.

5. Conclusion

In this study, we have utilized glioma patients along with two unique murine glioma models: RCAS glioma model and Gl261 model to study the BMDCs during different stages of glial tumor. Importantly, we identified the unique the population VEGFR2+MDSCs in both patients and mice, which might be used as a surrogate marker for glioma diagnosis and prognosis in future. We have validated the changes of myeloid lineage and endothelial lineages while the progression of gliomas, and observed the increased population of myeloid derived suppressor cells and endothelial progenitor cells in murine glioma models. We have created inducible VEGFR2 knockout system in glioma bearing mice. Taking advantage of this transgenetic model, we demonstrated that bone marrow derived VEGFR2 signaling plays an important role in myeloid differentiation, and infiltration into tumor tissues. Deficiency of VEGFR2 in BMDCs led to impairment of tumor associated myeloid cells and delayed progression of low-grade glioma. All of these findings may help to find the approach to suppress the progression of low-grade glioma into high-grade form, and have implications to predict the long-term survival of glioma patients [13,14].

We have studied the PDGFRα+ and VEGFR2+ lineages of BMDCs in both low-grade and high-grade glioma patients. We validated functional role of VEGFR2+ BMDCs in malignant transformation, and investigate the connection of myeloid differentiation with tumor associated macrophages/neutrophils. We delineated the signaling pathways, which affect pro-tumoral myeloid cells, and further characterize the downstream signaling of inhibitor of DNA binding proteins 2 (ID2) in BMDCs [15,16]. Deficiency of ID2 in BMDCs led to the down-regulation of VEGFR2, suppression of pro-angiogenic myeloid cells and prevention of low-grade to high-grade transition. Tumor-secreted TGF-β and GM-CSF are shown to enhance the VEGFR2/ID2 signaling axis in BMDCs. Targeting this axis could restrict the generation of tumor-associated myeloid cells and prevent malignant transformation in patients with pre-malignant gliomas, and suggests a role for therapeutic modulation of VEGFR2/ID2 signaling.

6. Publications, Abstracts, and Presentations

1) Abstract/Oral presentation

ID2/KDR drives the differentiation of pro-malignant myeloid derived suppressor cells in glioma. Yujie Huang, Prajwal Rajappa, Jacqueline Bromberg, David Lyden, Jeffrey Greenfield. Cold Spring Harbor-Asia Meeting (International) "FRONTIERS OF IMMUNOLOGY IN HEALTH & DISEASES" September 2–September 6, 2014

ID2/KDR drives formation of pro-malignant myeloid derived suppressor cells in glioma.
Yujie Huang, Prajwal Rajappa, Jacqueline Bromberg, David Lyden, Jeffrey Greenfield.
Annual Scientific Meeting of the Society for Neuro-Oncology, November 13-16, 2014

Oligodendrocyte progenitor cells promote neovascularization in glioma by disrupting the blood-brain barrier. Huang Y, Hoffman C, Rajappa P, Kim JH, Hu W, Huse J, Tang Z, Li X, Weksler B, Bromberg J, Lyden DC, Greenfield JP. Cancer Research. 2014 15;74(4):1011-21.

Restriction of Early Myeloid Lineage Directs Malignant Progression of Glioma. Huang Y, Rajappa P, Hu W, Hoffman C, Cisse B, Kim JH, Gorge E, Cope W, Vartanian E, Xu R, Zhang T, Yanowitch R, Weksler B, Pisapia D, Xiang J, Huse J, Matei I, Peinado H, Bromberg J, Holland E, Ding B, Rafii S, Lyden D, Greenfield J. Journal of Clinical Investigation. (Under Revision)

7. Inventions, Patents and Licenses

None

8. Reportable Outcomes

None

9. Other Achievements

None

10. References

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11. Training & Professional Development

I have received extensive trainings and related proceedings from various workshops, meetings, and hands-on practices, in addition to regular mentorships form weekly lab meeting and journal club. To follow the frontiers of tumor immunology and tumor microenvironment research, I have attended a workshop on tumor microenvironment (TME) organized by national cancer institute in April 2014. I have communicated several renowned experts on TME with their study and our proceedings. I also delivered a talk about our work in Cold Spring Harbor-Asia meeting focusing on immunology in diseases, and it was well received. In November 2014, my abstract on protumora myeloid differentiation was selected for oral presentation on Annual Meeting of Society of Neuro-Oncology. Part of my research on PDGF signaling contributed to a publication on Cancer Research in 2014, and the major part of this work is under revision as manuscript submitted to Journal of Clinical Investigation. Additionally, to update my knowledge on biomedical and genomic fields, I have continued courses and workshops including "Genomic workshop", "Next generation sequencing analysis", and "Biostatics for Clinical Studies", which are provided by Clinical Translational Science Center in Weill Cornell Medical College. All the training opportunities armed me for better bench-side research and long-term career development. According to the feedback from my presentations and publications, our work has been well received by scientific community. I am confident to achieve the career goal that I set in the original proposal.

Appendices - N/A